

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Mikhail Markovich GUSYATINER, et al.  
SERIAL NO: NEW APPLICATION  
FILED: HEREWITH  
FOR: L-ARGININE PRODUCING ESCHERICHIA COLI AND METHOD OF PRODUCING L-ARGININE

GAU:  
EXAMINER:



REQUEST FOR PRIORITY

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

- ☐ Full benefit of the filing date of U.S. Application Serial Number, filed, is claimed pursuant to the provisions of **35 U.S.C. §120.**
- ☐ Full benefit of the filing date of U.S. Provisional Application Serial Number, filed, is claimed pursuant to the provisions of **35 U.S.C. §119(e).**
- ☒ Applicants claim any right to priority from any earlier filed applications to which they may be entitled pursuant to the provisions of **35 U.S.C. §119**, as noted below.

In the matter of the above-identified application for patent, notice is hereby given that the applicants claim as priority:

<u>COUNTRY</u>	<u>APPLICATION NUMBER</u>	<u>MONTH/DAY/YEAR</u>
Russia	2000117677	July 6, 2000

Certified copies of the corresponding Convention Application(s)

- ☒ are submitted herewith
- ☐ will be submitted prior to payment of the Final Fee
- ☐ were filed in prior application Serial No. filed
- ☐ were submitted to the International Bureau in PCT Application Number .  
Receipt of the certified copies by the International Bureau in a timely manner under PCT Rule 17.1(a) has been acknowledged as evidenced by the attached PCT/IB/304.
- ☐ (A) Application Serial No.(s) were filed in prior application Serial No. filed ; and  
(B) Application Serial No.(s)
  - ☐ are submitted herewith
  - ☐ will be submitted prior to payment of the Final Fee

Respectfully Submitted,

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(РОСПАТЕНТ)



**ФЕДЕРАЛЬНЫЙ ИНСТИТУТ ПРОМЫШЛЕННОЙ СОБСТВЕННОСТИ**

рег. No 20/12-285

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### СПРАВКА

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**Название изобретения**

L-Arginine producing Escherichia coli  
and Method of Producing L-Arginine

**Заявитель**

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Уполномоченный заверить копию  
заявки на изобретение

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L-Arginine producing *Escherichia coli* and Method of  
Producing L-Arginine

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Background of the Invention

Field of the Invention

The present invention relates to L-arginine  
producing *Escherichia coli* and a method of producing L-  
10 arginine by fermentation using *Escherichia coli*. L-  
arginine is an industrially useful amino acid as  
ingredients of liver function promoting agents, amino  
acid transfusions, comprehensive amino acid preparations  
and the like.

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Description of the Related Art

It is known that some mutants of *Escherichia coli*  
resistant to analogs of arginine and pyrimidines produce  
arginine (Pierard A. and Glansdorf N., *Mol. Gen. Genet.*,  
20 118, 235, 1972. and Glansdorf N., *Biosynthesis of*  
arginine and polyamines. In "*E. coli* and *Salm.*  
*thyphimurium*, 1996). Additionally, the methods for  
producing arginine using mutants of *E. coli* resistant to  
some other drugs or recombinant strain of *E. coli* into  
25 which a gene encoding an enzyme of arginine biosynthetic  
pathway is introduced are known.

In arginine biosynthetic pathway of *E. coli* &

one mole of acetyl-CoA is consumed and one mole of acetic acid is released to produce one molecule of arginine (Fig. 1). As a result of the acetate by-production, a significant part of carbon source is wasted, besides, the accumulation of acetate worsens the growth of culture of arginine producers.

It is also known that *E. coli* can not effectively utilize acetate as carbon source.

### Summary of the Invention

From the above point of view, the present inventors conceived that the arginine production would be higher, if strains producing arginine had ability to re-utilize acetic acid. Accordingly, an object of the present invention is to provide arginine producing strain of *E. coli* which utilize acetic acid and a method for producing arginine using the strain.

Then the inventors constructed mutants of *E. coli* arginine producer which can utilize acetic acid and have succeeded in improving the arginine productivity of the *E. coli* arginine producer. Thus the present invention has been accomplished.

That is, the present invention provides *Escherichia coli* which has an ability to produce arginine and an ability to utilize acetate.

The present invention further provides a method

producing arginine comprising the steps of cultivating *E. coli* strain which has an ability to utilize acetate and an ability to produce arginine, in a culture medium to produce and accumulate arginine in the medium, and  
5 collecting arginine from the medium.

In the present invention, an amino acid is of L-configuration.

The present invention will be explained in detail below.

10 *E. coli* of the present invention has an ability to utilize acetate and an ability to produce arginine. *E. coli* strain having the ability to utilize acetate and the ability to produce arginine may be obtained by imparting the ability to utilize acetate to *E. coli*  
15 strain having the ability to produce arginine, or by imparting the ability to produce arginine to *E. coli* strain having the ability to utilize acetate.

For the present invention, the term "ability to produce arginine" refers to an ability of *E. coli* strain used for the present invention to produce and accumulate  
20 arginine in a medium when the *E. coli* strain is cultivated in the medium. The term "ability to utilize acetate " refers to an ability to metabolize acetic acid or acetate more efficiently than the parental strain  
25 for example, an ability of *E. coli* strain used for the present invention to grow faster than the parental strain when the *E. coli* strain is cultivated in a medium.

containing acetic acid or acetate as a sole carbon source. More concretely, it can be said that *E. coli* strain has ability to utilize acetate if the strain grows faster than the parental strain when the strains are cultivated in a medium containing acetic acid or acetate as a sole carbon source, for example, the liquid minimal medium A (described below) containing 5 g/L of ammonium acetate under an appropriate condition. Most concretely, it can be said that *E. coli* strain has ability to utilize acetate if the strain forms a colony within 2 days at 37°C when the strain is cultivated on an agar medium containing acetic acid or acetate as a sole carbon source, for example, the minimal medium A (described below) containing 5 g/L of ammonium acetate and agar under an appropriate condition. The term "an appropriate condition" refers to temperature, pH, air supply or optional presence of essential nutrients or the like for the *E. coli* strain which is to be cultivated.

As an example of a method for obtaining *E. coli* of the present invention, a method of inducing a mutant having an ability to utilize acetate from *E. coli* strain having an ability to produce arginine will be explained below.

*E. coli* having an ability to produce arginine is not specifically limited, provided that it can be imparted an ability to utilize acetate. Such *E. coli*

strains include arginine-producing strains bred from *E. coli* K-12, B, C, or those derivatives.

As examples of the *E. coli* arginine producer, the following may be mentioned: a mutant having resistance to  $\alpha$ -methylmethionine, *p*-fluorophenylalanine, D-arginine, arginine hydroxamate, *s*-(2-aminoethyl)-cysteine,  $\alpha$ -methylserine,  $\beta$ -2-thienylalanine or sulfaguanidine (Japanese Laid-Open Publication No. 56-106598), arginine-producing strain into which *argA* gene encoding N-acetylglutamate synthetase is introduced (Japanese Laid-Open Publication No. 57-5693) and the like. *E. coli* strain 237 which is described in after-mentioned Examples is also preferable arginine-producing strain. The strain 237 has been deposited in All-Russian Collection for Industrial Microorganisms (VKPM) under the accession number VKPM B-7925 since April 10, 2000.

A mutant strain having an ability to utilize acetate may be obtained from an arginine-producing strain as described above by, for example, mutagenizing the arginine-producing strain and selecting strains which can grow in a minimal medium containing acetic acid or acetate as a sole carbon source. Mutagenesis can be performed by, for example, UV irradiation or with an agent usually used for artificial mutagenesis such as methyl-3-nitro-1-nitrosoguanidine (NTG) and nitrous acid. Mutagenesis and selection of a mutant strain having an ability to utilize acetate may be repeated two or more

times.

Arginine can be efficiently produced by cultivating the *E. coli* strain described above, which has the activity to utilize acetate and to produce arginine, in a culture medium to produce and accumulate arginine in the medium, and collecting arginine from the medium.

Acetylation of glutamate to N-acetylglutamate and deacetylation of N-acetylornithine to ornithine in arginine biosynthesis of coryneform bacteria are catalyzed by the same enzyme, ornithine acetyltransferase. On the other hand, acetylation and deacetylation in arginine biosynthesis of *E. coli* are catalyzed by different enzymes, N-acetylglutamate synthase and N-acetylornithinase, respectively. Therefore, if by-produced acetic acid would be utilized, its effect on arginine production has been unknown.

In the method for producing arginine of present invention, the cultivation of *E. coli*, the collection and purification of arginine from the liquid medium may be performed in a manner similar to the conventional fermentation method wherein arginine is produced using *E. coli*.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as acetic acid, fumaric acid, citric



acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic  
5 nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B<sub>1</sub> and L-isoleucine or yeast extract to be contained in appropriate amounts as organic trace  
10 nutrients. Other than the above, potassium phosphate magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

Cultivation is preferably carried out under an aerobic condition for 16-72 hours. The cultivation  
15 temperature is controlled at 25°C to 45°C, and pH is controlled at 5-8 during cultivation. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment.

Collection of arginine from fermented liquor is  
20 usually carried out by combining an ion exchange resin method and other known methods.

#### Best Mode for Carrying out the Invention

25 Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Induction of acetate utilizing mutants

From the arginine-productive mutant *E. coli* strain  
5 237, mutants which grew well on the M9 agar medium  
containing ammonia acetate (5 mg/ml) as a sole carbon  
and nitrogen source were induced. The strain 237 is a  
mutant resistant to a pyrimidine analog, 6-azauracil  
which was induced from *E. coli* K12 *ilvA::Tn5* by using  
10 methyl-3-nitro-1-nitrosoguanidin (NTG). The strain 237  
grows poorly on M9 agar containing ammonia acetate as a  
sole carbon and nitrogen source. The strain 237 has  
been deposited in the All-Russian Collection for  
Industrial Microorganisms (VKPM) under accession number  
15 VKPM B-7925.

Cells of the strain 237 were grown overnight in L-  
broth with shaking (test-tube, 37°C) and harvested by  
centrifugation. Then, the cells were resuspended in  
saline solution (0.8%) containing 0.1 mg/ml of NTG.  
20 After 30 min of exposure to NTG at 37°C the cells were  
spun down, washed twice with saline and plated on the  
minimal agar medium A, containing 5 g ammonia acetate, 6  
g  $\text{Na}_2\text{HPO}_4$ , 3 g  $\text{KH}_2\text{PO}_4$ , 0.5 g NaCl, 0.1 mg thiamin, 0.1 g  
L-isoleucine, 15 g agar, per 1 liter of water (pH 7.0).

25 The plates were incubated for 5 days at 37°C.  
Colonies appeared within 2 days on the plates were  
picked up and purified by streaking on the same agar.

plates. The parental strain 237 formed colonies only after 5 day's cultivation. Frequency of acetate utilizing mutant was  $6 \times 10^{-5}$ . Seventy purified strains were tested for their productivity of arginine. About 1/4 of the mutants derived was more productive than parent strain 237. The best arginine producer among them was strain 382. The strain 382 has been deposited in All-Russian Collection for Industrial Microorganisms (VKPM) under the accession number VKPM B-7926 since April 10, 2000.

Example 2: Growth of the novel mutants on acetate

Two ml portions of the liquid minimal medium A (agar was not added), containing either ammonia acetate (5 g/L) or glucose (5 g/L) as sole carbon sources, were placed into test tubes, inoculated with one loop of the novel strain 382, another example of arginine producing mutant, strain 383 and their parent strain 237, and incubated for 16 hours at 32 C with shaking. The growth was determined by measuring optical density of the culture at 540 nm. The Optical density of the media at the start of the culture was about 0.05. The results are shown in Table 1.

Table 1

Strain	Growth (OD <sub>540</sub> ) in liquid minimal medium For 16 hours with:	
	Glucose (0.5%)	Ammonia acetate(0.5%)
237 (parent)	1.8	0.4
382	1.5	1.0
383	1.6	0.7

Example 3: Production of arginine by the novel arginine producing mutants in test-tube fermentation

5           The novel strain 382, 383 and their parent strain 237 were cultivated in the fermentation medium. The fermentation medium contained 60g glucose, 25g ammonium sulfate, 2g  $\text{KH}_2\text{PO}_4$ , 1g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 mg thiamine, 5g yeast extract (Difco), 25g calcium carbonate, per 1 liter of tap water (pH 7.2). Glucose and chalk were sterilized separately. Two ml portions of the medium were placed into test-tubes, inoculated with one loop of the tested microorganisms, and the cultivation was carried out at 32°C for 3 days with shaking. The accumulated amount of arginine in the culture medium is shown in Table 2.

Table 2

Strain	Arginine (g/L)
237 (parent)	5.1
382(acetate utilizing mutant)	12.0
383(acetate utilizing mutant)	7.7

20           Example 4: Production of arginine by the novel arginine producing mutant in jar fermenter

The novel strain 382 and its parent strain 237 were cultivated with shaking at 32°C for 8 hours in L-broth. Then, 60 ml of the resulted seed culture were inoculated into 1 liter jar fermenter containing 0.5 L of the a fermentation media, followed by culturing with stirring at 700 rpm at 32° C and aeration rate 0.5 liter/min. The fermentation medium contained 100g glucose, 9g ammonia sulfate, 1g  $\text{KH}_2\text{PO}_4$ , 0.4g  $\text{MgSO}_4$ , 0.02g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02g  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g total nitrogen of Soybean hydrolysate, 0.3g L-isoleucine, 0.4mg thiamine in 1 liter of tap water (pH 7.0). During the cultivation, ammonia solution (4.7 M) was added to adjust pH to 7.0 and to supply a nitrogen source. The cultivation was carried out for 42 hours. The accumulated amount of arginine in the culture medium and the yield from glucose are shown in Table 3.

Table 3

Strain	Arginine (g/L)	Yield from glucose (%)
237 (parent)	4.5	5.2
382 (acetate utilizing mutant)	19.3	23.9

The acetate-utilizing mutant showed higher productivity of arginine than parental strain.

What is claimed is:

1. *Escherichia coli* which has an ability to produce arginine and an ability to utilize acetate.

5 2. *Escherichia coli* according to claim 1, wherein the bacterium forms a colony within 2 days at 37°C when the bacterium is cultivated on an agar medium containing acetic acid or acetate as a sole carbon source.

10 3. *Escherichia coli* according to claim 1 or 2 wherein the bacterium is a derivative of *Escherichia coli* K12.

15 4. A method of producing arginine comprising the steps of cultivating *E. coli* according to any of claims 1 to 3 in a culture medium to produce and accumulate arginine in the medium, and collecting arginine from the medium.

## Abstract

Arginine can be efficiently produced by cultivating *Escherichia coli* which has an ability to  
5 produce arginine and an ability to utilize acetate in a  
culture medium to produce and accumulate arginine in the  
medium, and collecting arginine from the medium.